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13. ABSTRACT (Maximum 200 Words)

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy, including advanced prostate cancer. We have focused on events related to cell cycle arrest (G1 and G2/M) and induction of apoptosis in human prostate cancer cells. Treatment with 2-ME increased cyclin B1 protein and its associated kinase activity followed by later inhibition of cyclin A-dependent kinase activity and induction of apoptosis. Similar results were obtained with paclitaxel (taxol), a clinically relevant agent used to treat advanced prostate cancer. Cyclin-dependent kinase inhibitors prevented 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity and blocked induction of apoptosis. Reduction of X-linked inhibitor of apoptosis (XIAP) protein by 2-ME and paclitaxel correlated with increased apoptosis. Lower doses of 2-ME and paclitaxel resulted in G1 (but not G2/M) cell cycle arrest in the p53 wild type LNCaP cell line, but with minimal induction of apoptosis. We suggest that 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires activation of cyclin B1-dependent kinase that arrests cells in G2/M and subsequently leads to the induction of apoptotic cell death.

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INTRODUCTION

One of the more promising emerging chemotherapeutic agents is 2-methoxyestradiol (2-ME), an endogenous metabolite of estradiol [1-3]. 2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer (AI-PC) [4,5] utilizing a remarkable number of diverse mechanisms that include mitotic cell cycle arrest and induction of apoptosis [1-3]. 2-ME's anti-prostate cancer activity, however, is poorly understood. A better understanding of the mechanisms of 2-ME's anti-prostate cancer effects will be helpful to better evaluate its clinical potential in managing AI-PC. 2-ME may be an example of a chemotherapeutic agent that takes advantage of the molecular and biochemical differences between cancer and normal cells. One such difference may be the requirement for cell cycle proteins like cyclins and cyclin-dependent kinases. Our hypothesis is that one of the cancer-specific mechanisms whereby 2-ME exerts its anti-prostate cancer activity is the deregulated activation of cyclin B1/cdc2 kinase during the cell cycle, which results in the induction of apoptotic cell death. The purpose and scope of this research proposal is to (1) determine the molecular mechanisms of the 2-ME-mediated G2/M cell cycle arrest in prostate cancer cell lines; (2) determine whether activation of cyclin B1/cdc2 kinase by 2-ME is required for induction of apoptosis in prostate cancer and non-transformed normal cells; and (3) identify synergisms and mechanisms of interaction between 2-ME and other clinically relevant chemotherapeutic drugs. In this annual report, we present our accomplishments in the first year of the proposal.

BODY

To better understand 2-ME's anti-prostate cancer action, we have focused on events related to mitotic cell cycle arrest (G2/M) and induction of apoptosis in LNCaP, DU 145, and PC-3 human prostate cancer cell lines. A manuscript in press entitled "2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells" summarizes some of our results and is included in the appendix. Blocking the 2-ME and paclitaxel increase in cyclin B1/cdk activity with the potent cdk inhibitors purvalanol A and alsterpaullone resulted in decreased apoptosis (see Fig. 9, appended manuscript). These results suggest that 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity is required for induction of apoptosis in prostate cancer cells.

The following sections will report our findings associated with each task in the approved statement of work.

Specific Aim 1: Determine the molecular mechanisms of the 2-ME/2-EE-mediated G2/M cell cycle arrest in prostate cancer cell lines (months 1-30).

1. Determine the effect of 2-ME/2-EE on the cdc2 phosphorylation status of thr-14 and thr-161 positions in the human prostate cancer cell lines LNCaP, DU 145, and PC-3 using quantitative Western blot (months 1-4).

Completed and presented last year in the annual report.

2. Identify quantitative differences in the total levels of cdc25C, myt1, wee1, and CAK proteins in 2-ME/2-EE treated prostate cancer cell lines using quantitative Western blot (months 1-4).

Completed and presented last year in the annual report.

3. Determine the effect of the novel anti-cancer cdc25C inhibitor MX7174 and the wee1 inhibitor PD0166285 on 2-ME/2-EE-mediated G2/M cell cycle arrest in prostate cancer cell lines using quantitative Western blot and flow cytometry (months 2-6).

We have not yet made progress in this task.

4. *Determine the effect of 2-ME/2-EE on the subcellular localization of cyclin B1 and the other regulators of cdc2 kinase using Western blot and immunocytochemistry (months 2-6).*

Nuclear and cytoplasmic protein lysates were prepared from LNCaP and DU 145 cells treated with control and 5 μM 2-ME for 24h and analyzed for cyclin B1 and cdk1 expression by Western blot analysis (**Fig. 1**). Cyclin B1 was found to be predominantly localized in the nuclear compartment in control and 2-ME treated DU 145 cells, whereas cdk1 was localized in both nuclear and cytoplasmic compartments. In LNCaP cells, in which 2-ME induces greater amounts of apoptosis compared to DU 145 cells, there were greater amounts of cyclin B1 protein in the cytoplasmic lysate upon treatment with 2-ME compared to control cells; cdk1 levels were similar in both nuclear and cytoplasmic lysates (**Fig. 1**). These results need to be confirmed by immunocytochemical staining of cyclin B1 and microscopic analysis. If deregulated cyclin B1/cdk1 activity is important in the induction of apoptosis, a prediction is that it should be active in the cytoplasmic fraction, where proteins regulating apoptosis are likely to be located.

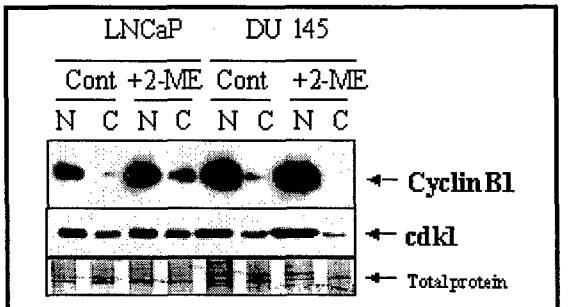

Fig. 1. Western blot analysis of cyclin B1 and cdk1 expression in nuclear (N) and cytoplasmic (C) lysates prepared from LNCaP and DU 145 cells treated with 5 μM 2-ME or control for 24h. Total protein from Commassie blue stain is shown as loading control.

5. *Develop and characterize stable LNCaP, DU 145, and PC-3 Tet-Off inducible cell lines containing the dn-cdc2, cdc2-AF, and cyclin B1-AS genes regulated by the addition (off) or removal (on) of dox in the media (months 4-24).*

This task is in progress. We have cloned the dominant negative cdk1 cDNA into the pTRE-Tight vector from Clontech and have co-transfected this plasmid with the pTK-Hyg hygromycin selection plasmid into the LNCaP-Tet-Off cell line (from Kerry Burnstein). The pTRE-Tight vector offers the advantage of reduced basal level expression and increased overall induction of the gene of interest.

6. *Determine the effect of expressing dn-cdc2, cdc2-AF, and cyclin B1-AS on 2-ME/2-EE-mediated G2/M arrest in LNCaP, DU 145, and PC-3 Tet-Off inducible cell lines using flow cytometry (months 8-30).*

Not yet started.

Is the 2-ME-mediated mitotic block required for induction of apoptosis? In addition to the above tasks, we undertook an important experiment to determine if 2-ME mediated block in the G2/M phase of the cell cycle is required for apoptosis. We used aphidicolin to block the cell cycle at the S phase. Flow cytometric analysis showed the expected increase in S and decrease in G2/M phase when LNCaP and DU 145 cells were treated with 1 μM aphidicolin for 24h (**Fig. 2**). To address the effect on 2-ME mediated apoptosis, cells were treated for 24 h with aphidicolin (to block in S) followed by the addition of 5 μM 2-ME for an additional 48 h (72 h total time). Flow cytometric analysis showed no increase in G2/M in these conditions

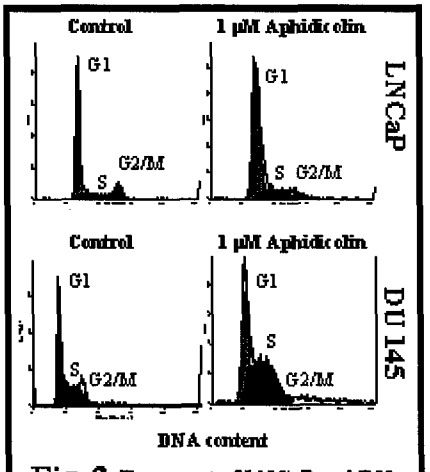

Fig. 2. Treatment of LNCaP and DU 145 cells with 1 μM aphicicolin for 24h blocked in S and reduced G2/M.

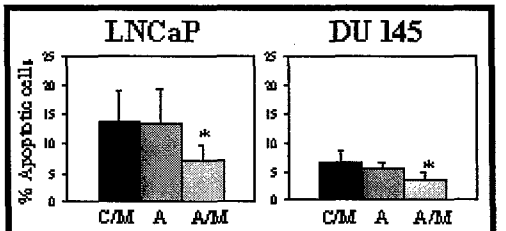

Fig. 3. Synchronizing LNCaP and DU 145 cells with aphidicolin significantly reduced 2-ME-mediated apoptosis as determined by DAPI apoptosis assay (n=5; *, $P<0.05$).

(not shown). We analyzed for apoptotic cells using the DAPI assay and compared LNCaP and DU 145 cells treated with control (0.1% DMSO) for 24 h followed by 5 μM 2-ME for 48 h (C/M), 1 μM aphidicolin for 72 h (A), and aphidicolin (72 h) plus 2-ME (last 48 h of 72 h incubation) (A/M). The results showed a significant decrease ($P<0.05$, Student's *t*-test) in apoptotic cells in A/M compared to C/M and A in LNCaP and DU 145 cells (**Fig. 3**). These results suggested that 2-ME mediated G2/M block was important for increased apoptosis.

Novel analogs of 2-ME inhibits the growth of prostate cancer cells. To determine the effect of 2-ME and 3 novel analogs (ENMD-1237, -1200, and -1198 are more resistant than 2-ME to inactivation by metabolism) on prostate cancer cell viability, we performed a 3-day cell growth assay using the CellTiter Aqueous cell proliferation colorimetric method (Promega, Madison, WI), as per manufacturer's instructions. Cell viability was normalized against the vehicle control and the data expressed as a percentage of control from three independent experiments done in triplicate. Experiments using 2-ME and 2-ME analogs 1237, 1200, and 1198 (obtained from EntreMed) showed that 1198 was the most effective drug inhibiting the growth of PC-3 cells (IC_{50}: 2-ME=1.1 μM; 1237=1.2 μM; 1200=0.11 μM; 1198=0.06 μM) (**Fig. 4**). *Overall, these results suggest that 1198 and 2000 were the best 2-ME analogs to inhibit the growth of prostate cancer cells.*

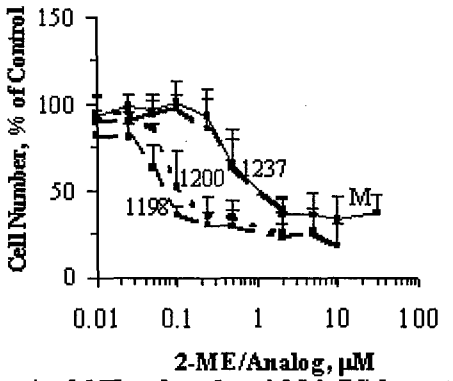

Fig. 4. 2-ME and analogs inhibit PC-3 prostate cancer cells. PC-3 human prostate cancer cell line was treated with 2-ME and analogs 1198, 1200, and 1237 (0.1-30 μM) or vehicle control and cell viability was measured using the CellTiter Aqueous colorimetric method. 2-ME analogs 1198 and 1200 inhibit PC-3 cells more effectively than 2-ME (M) or 1237.

<u>Specific aim 2:</u> Determine whether activation of cyclin B1/cdc2 kinase by 2-ME/2-EE is required for induction of apoptosis in the Tet-Off inducible prostate cancer cell lines and in stably transfected non-transformed normal cells (months 1-30).

1. Determine whether 2-ME/2-EE treatment of the non-transformed/normal cell lines (BPH-1, NRP-152, primary prostate, CD34+ bone marrow progenitor) results in G2/M arrest and apoptosis (flow cytometry), and correlate with the expression levels of cyclin B1 protein (quantitative Western blot) (months 1-24).

Completed and presented last year in the annual report.

2. Use Tet-Off inducible prostate cancer cell lines to determine if inhibition of cdc2 kinase with dn-cdc2, cyclin B1-AS, and MX7174 will decrease 2-ME/2-EE-mediated apoptosis (months 8-30).

We are waiting for the identification of Tet-Off inducible cell lines before starting this task.

3. Use Tet-Off inducible prostate cancer cell lines to determine if further activation of cdc2 kinase with cdc2-AF and PD0166285 will increase 2-ME/2-EE-mediated apoptosis (months 8-30).

We are waiting for the identification of Tet-Off inducible cell lines before starting this task.

4. Determine whether stable expression of cyclin B1 in NRP-152 and MSC sensitizes them to 2-ME/2-EE-mediated apoptosis (months 4-24).

This task is in progress. We previously attempted to obtain an NRP-152 stable line expressing cyclin B1 but failed because we used an expression plasmid that did not function correctly. We now have a functional cyclin B1 expression plasmid and should have results for the final report next year.

5. *Determine whether stable expression of cyclin B1-AS in BPH-1 will reduce cyclin B1 protein levels and decrease 2-ME/2-EE-mediated apoptosis (months 4-24).*

Reduction of cyclin B1 protein by siRNA decreases 2-ME-mediated induction of apoptosis in DU 145 prostate cancer cells. To determine if reduction of cyclin B1 protein has an effect on 2-ME-mediated apoptosis, we used siRNA SMARTpool specific for cyclin B1 (Dharmacon, Lafayette, CO). LNCaP cells were seeded in 12 well plates and transfected with 50 nM of siRNA (cyclin B1 and siCONTROL non-targeting pool [Dharmacon]) with oligofectamine (Invitrogen, Carlsbad, CA) for 48 h, following the manufacturer's instructions. The results showed a 7-fold reduction in cyclin B1 protein in LNCaP cells transfected with cyclin B1 siRNA compared to control siRNA (**Fig. 5A**). Subsequently, we transfected LNCaP cells with cyclin B1 and control siRNA for 48 h, re-seeded the cells, and re-transfected the next day (to maintain low cyclin B1 protein levels) in the presence or absence of 5 μM 2-ME for 48 h. The results showed that in the presence of cyclin B1 siRNA, there was less cleavage of PARP in 2-ME treated cells compared to control siRNA (**Fig. 5B**). These results suggested that higher cyclin B1 protein levels can sensitize prostate cancer cells to 2-ME-mediated apoptosis.

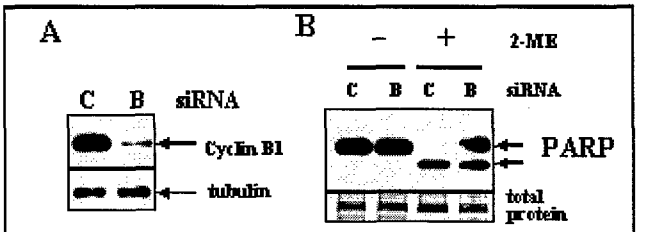

Fig. 5. Reduction of cyclin B1 protein by siRNA reduces 2-ME mediated apoptosis. **A.** Western blot analysis of cyclin B1 showing lower levels in LNCaP cells treated with siRNA specific for cyclin B1 (B) compared to control siRNA (C). Tubulin is loading control. **B.** Western blot analysis showing less 2-ME mediated cleavage of PARP in LNCaP cells treated with cyclin B1 siRNA (B) compared to cells treated with control siRNA (C). Total protein is loading control.

2-ME increases apoptosis in PC-3/AR (overexpresses cyclin B1) prostate cancer cells. We investigated whether PC-3/AR prostate cancer cells (express AR) are more sensitive to 2-ME-mediated apoptosis. The results showed that treatment of the PC-3/AR cells with 5 μM 2-ME for 3 and 6 days resulted in 2- to 6-fold greater apoptosis compared to PC-3/neo negative control cells (**Fig. 6**). Interestingly, there is ~5-fold more cyclin B1 protein expressed in PC-3/AR compared to PC3/neo cells, suggesting that overexpression of cyclin B1 sensitizes these cells to 2-ME-mediated apoptosis (**Fig.7**). Treatment of PC-3/AR cells with cyclin B1 siRNA will better determine if this statement is correct.

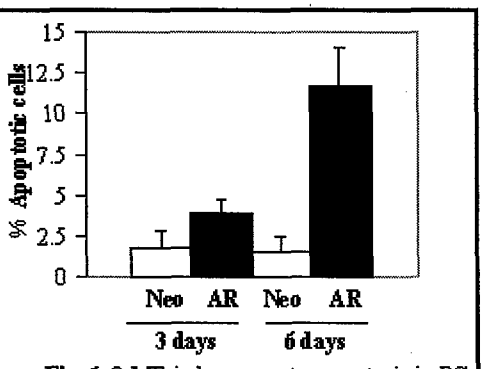

Fig. 6. 2-ME induces greater apoptosis in PC-3/AR compared to PC3/neo cells as determined by the DAPI apoptosis assay.

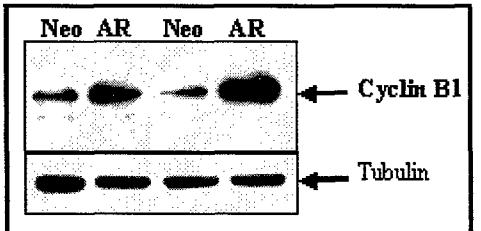

Fig. 7. Western blot analysis showing greater expression of cyclin B1 in PC-3/AR compared to PC-3/neo cells.

Specific aim 3: Identify synergisms and mechanisms of interaction between 2-ME/2-EE and other clinically relevant chemotherapeutic drugs (months 8-36).

1. Identify the in vitro growth condition (multicellular spheroids using polyhema) whereby prostate cancer cells are in a non-proliferative state and determine if 2-ME/2-EE will induce G2/M arrest and apoptosis (months 8-10).

2. Correlate the levels of cyclin B1 protein in the non-proliferation condition with the ability of 2-ME/2-EE to increase cdc2 kinase activity and induce G2/M arrest and apoptosis (8-10).

We presented some data in the last annual report and have not as of yet made progress. We plan on pursuing these tasks and have more data for the final report.

3. Determine the IC_{50} dose and the effect on the cell cycle for docetaxel (taxotere), R-roscovitine, and etoposide in prostate cancer cell lines (months 10-14).

We presented data for docetaxel inhibition of prostate cancer cell lines in the last annual report.

4. Use isobolograph analysis of cell growth assays to determine if docetaxel, R-roscovitine, and etoposide can synergize with 2-ME/2-EE to inhibit prostate cancer cells in proliferating and non-proliferating in vitro conditions (months 14-18).

5. Determine whether the drug that can act synergistically with 2-ME/2-EE to inhibit prostate cancer cell growth also has a synergistic effect on the cell cycle and apoptosis (months 16-20).

Combination of 2-ME and docetaxel increases apoptosis in PC-3/AR prostate cancer cells. We investigated whether 2-ME and docetaxel can increase apoptosis greater than each drug alone in PC-3/AR prostate cancer cells. The results showed that treatment with 5 μM 2-ME and 10 nM docetaxel for 72 h increased the number of apoptotic cells over each drug alone (**Fig. 8**). These results suggested that 2-ME and docetaxel may be an effective drug combination in AI-PC. We are currently using the CellTiter Aqueous cell proliferation colorimetric method to determine if the 2-ME and docetaxel growth inhibition of PC-3/AR (and other prostate cancer cell lines) are synergistic.

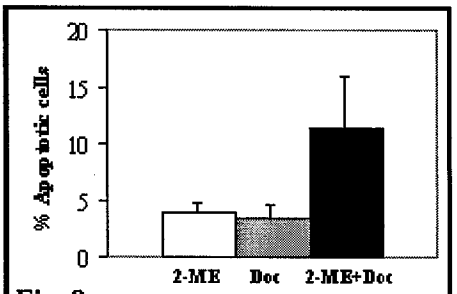

Fig. 8. DAPI apoptosis assay showing that 2-ME and docetaxel increased apoptosis in PC-3/AR prostate cancer cells greater than each drug alone.

6. Using the most promising drug combination identified in vitro, test the in vivo anti-prostate cancer efficacy in the Gγ/T-15 and TRAMP transgenic mice (10 mice per group x 6 groups = 60 Gγ/T-15 and 60 TRAMP transgenic male mice) (months 20-36).

2-ME and docetaxel combination inhibits primary and metastatic prostate tumors in Gγ/T-15 transgenic mice. Gγ/T-15 mice with palpable prostate tumors were randomly divided into experimental and control groups and injected i.p. every day (2-ME) or every 3 days (docetaxel) with 2-ME (M, 150 mg/kg; n=9), docetaxel (D, 5 mg/kg; n=6), combination of 2-ME and docetaxel (MD), or vehicle control (n=9) for two weeks. We used the new nanocrystal colloidal dispersion (NCD) liquid formulation developed by EntreMed for 2-ME, shown to significantly increase bioavailability and is now being tested in clinical trials. Primary prostate tumors and visible lymph node metastases were removed and weight determined. The results showed that treatment of mice with 2-ME and docetaxel inhibited primary prostate tumors by 35-40% (*, $P<0.05$) and metastatic prostate tumors by 57-65% (**Fig. 9**). Treatment of mice with the 2-ME and docetaxel combination (MD) resulted in a 52% inhibition of primary (**, $P<0.002$) and 85% inhibition of metastatic prostate tumors (*, $P<0.03$) (**Fig. 9**). There was a trend for greater inhibition of primary and metastatic prostate tumors with the 2-ME/docetaxel combination compared to each drug alone; however

there was not a statistical significance. These results in the Gγ/T-15 transgenic mice suggested that the 2-ME and docetaxel combination effect on primary prostate tumors was not additive to each drug alone but significantly inhibited metastases. We will determine the effect on proliferation and apoptosis. Currently, we are investigating whether the combination of lower doses of 2-ME and docetaxel are more effective in the Gγ/T-15 mice.

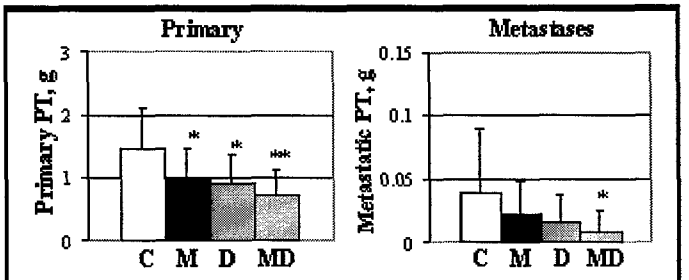

Fig. 9. 2-ME and docetaxel combination is more effective inhibiting primary and metastatic prostate tumors in Gγ/T-15 mice. Primary and metastatic prostate tumors were weighed after treatment with 2-ME (M) Docetaxel (D), M+D (MD) and compared with controls (C). *, $P<0.05$; **, $P<0.002$.

KEY RESEARCH ACCOMPLISHMENTS

- Treatment of LNCaP cells with 2-ME resulted in a greater cytoplasmic localization of cyclin B1 protein, possibly correlating to greater induction of apoptosis (Fig. 1).
- 2-ME requires arrest in G2/M for maximal induction of apoptosis in prostate cancer cells (Fig. 3).
- Novel analogs of 2-ME (ENMD-1198 and -1200) are more effective growth inhibitors of prostate cancer (Fig. 4).
- Reduction of cyclin B1 protein with siRNA protects prostate cancer cells from 2-ME-mediated apoptopsis (Fig. 5).
- PC-3/AR cells are more sensitive to 2-ME-mediated apoptosis compared to PC-3/neo cells, possibly due to greater expression of cyclin B1 (Figs. 6 and 7).
- Combination of 2-ME and docetaxel increases apoptosis in PC-3/AR cells greater than each drug alone; this effect is at least additive and possibly synergistic (Fig. 8).
- 2-ME and docetaxel combination inhibits primary and metastatic prostate tumors in Gγ/T-15 transgenic mice (Fig. 9).

REPORTABLE OUTCOMES

Publications

1. Brown JW, S Cappell, **C Perez-Stable**, and LM Fishman. 2004. Extracts from two marine sponges lower cyclin B1 levels, cause a G2/M cell cycle block and trigger apoptosis in SW-13 human adrenal carcinoma cells. Toxicon 43: 841-846.
2. **Perez-Stable CM.** 2005. 2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells. Cancer Letters, in press. **Appended manuscript.**

Abstracts

1. **Perez-Stable C**, T Reiner, and A de las Pozas. 2004. Preclinical testing of the taxotere and flavopiridol sequence combination in the Gγ/T-15 transgenic mouse model of androgen-independent prostate cancer. AACR Basic, Translational, and Clinical Advances in Prostate Cancer. Bonita Springs, FL November 17-21, 2004. Poster Presentation.
2. **Perez-Stable C**, A Gomez, and A de las Pozas. 2004. Sequence-specific treatment of LNCaP prostate cancer cells with flavopiridol followed by taxotere decreases XIAP and increases apoptosis. Society for Basic Urologic Research (SBUR). Savannah, GA. 12/9/04 - 12/12/04. Poster Presentation.

Employment Promotions

Dr. Perez-Stable was promoted to Research Associate Professor of Medicine at the University of Miami Miller School of Medicine, effective June 2004.

CONCLUSIONS

Our data supports the hypothesis that one of the cancer-specific mechanisms whereby 2-ME exerts its anti-prostate cancer activity is the deregulated activation of cyclin B1/cdc2 kinase during the cell cycle, which results in the induction of apoptotic cell death. First, reduction of cyclin B1 protein levels with siRNA provides specific genetic evidence that cyclin B1 can sensitize prostate cancer cells to 2-ME-mediated apoptosis. Second, the PC-3/AR cells, which overexpress cyclin B1 compared to PC-3/neo cells, are more sensitive to 2-ME-mediated apoptosis. In addition, prevention of 2-ME-mediated arrest in G2/M reduced apoptosis, suggesting that the increase in cyclin B1/cdc2 kinase activity in G2/M is important for induction of apoptosis. However, we will require more convincing evidence that cyclin B1 can sensitize prostate cancer cells to 2-ME-mediated apoptosis. We can also conclude that 2-ME and docetaxel can synergize together to induce apoptosis in PC-3/AR prostate cancer cells. In the Gγ/T-15 transgenic mouse model of prostate cancer, however, high doses of 2-ME and docetaxel do not inhibit the growth of prostate tumors in an synergistic or additive manner. Testing lower doses of 2-ME and docetaxel in the Gγ/T-15 mice are in progress.

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2-Methoxyestradiol and paclitaxel have similar effects on the cell cycle and induction of apoptosis in prostate cancer cells

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Abstract

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol with promise for cancer chemotherapy, including advanced prostate cancer. We have focused on events related to cell cycle arrest (G1 and G2/M) and induction of apoptosis in human prostate cancer cells. Treatment with 2-ME increased cyclin B1 protein and its associated kinase activity followed by later inhibition of cyclin A-dependent kinase activity and induction of apoptosis. Similar results were obtained with paclitaxel (taxol), a clinically relevant agent used to treat advanced prostate cancer. Cyclin-dependent kinase inhibitors prevented 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity and blocked induction of apoptosis. Reduction of X-linked inhibitor of apoptosis (XIAP) protein by 2-ME and paclitaxel correlated with increased apoptosis. Lower doses of 2-ME and paclitaxel resulted in G1 (but not G2/M) cell cycle arrest in the p53 wild type LNCaP cell line, but with minimal induction of apoptosis. We suggest that 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires activation of cyclin B1-dependent kinase that arrests cells in G2/M and subsequently leads to the induction of apoptotic cell death.

Keywords: Cyclin B1; Prostate cancer; Mitotic block; Apoptosis; Cyclin A

1. Introduction

One of the more promising emerging chemotherapeutic agents is 2-methoxyestradiol (2-ME), an endogenous metabolite of estradiol [1–4]. 2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer (AI-PC) utilizing a remarkable number of diverse mechanisms that include cell cycle arrest, induction of apoptosis, disruption of microtubules, inhibition of angiogenesis, and increasing oxidative damage [1–6]. What makes 2-ME a promising chemotherapeutic is that it does not harm quiescent or proliferating normal cells and it does not exert significant estrogenic effects from binding estrogen receptors [2,7]. In fact, because of 2-ME's anti-cancer

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activity without toxicity to normal cells, it is currently in Phase II human trials for breast and prostate cancer [3]. 2-ME's anti-prostate cancer activity, however, is not well understood. A better understanding of the mechanisms of 2-ME's anti-prostate cancer effects will be helpful to better evaluate its clinical potential in managing AI-PC.

One of the proposed mechanisms for 2-ME's anti-cancer effect is the disruption of microtubule function and subsequent block in the G2/M phase of the cell cycle [1,2,4]. We have previously shown that 2-ME inhibits both androgen-dependent LNCaP and androgen-independent DU 145 and PC-3 human prostate cancer cells independent of the expression of androgen receptor and tumor suppressors p53 and Rb [5]. 2-ME blocks LNCaP, DU 145, and PC-3 prostate cancer cells in the G2/M phase of the cell cycle and induces apoptosis [5,6]. Specific mechanisms for 2-ME induced inhibition in prostate cancer cells are proposed to be mediated by activation of c-Jun N-terminal kinase (JNK) and inactivation of the anti-apoptosis proteins Bcl-2/Bcl-xL [8–10], up-regulation of the death receptor 5 and induction of the extrinsic pathway of apoptosis [11], and down-regulation of hypoxia-inducible factor-1 [12]. The effect of 2-ME on the components of the cell cycle, specifically the G2/M cyclins A and B1, and whether these effects are required for induction of apoptosis are not known.

Paclitaxel is a well studied chemotherapeutic agent that stabilizes microtubules and has clinical efficacy in a variety of cancers, including AI-PC [13]. Paclitaxel-mediated microtubule damage activates the mitotic checkpoint and blocks the degradation of cyclin B1, leading to a prolonged activation of cyclin B1-dependent kinase (cdk)1 and mitotic arrest [14–16]. The prolonged activation of cdk1 is required for paclitaxel-mediated apoptosis in the MCF-7 breast cancer cell line, as demonstrated by the use of the chemical inhibitor of cdk1, olomoucine, and anti-sense oligonucleotides specific for cyclin B1 [14]. It appears, however, that the subsequent reduction of cyclin B1-cdk1 activity and exit from the paclitaxel-mediated mitotic block is important for induction of apoptosis [17]. The mechanism proposed is that increased cdk1 activity results in phosphorylation and stabilization of survivin, a member of the inhibitor of apoptosis (IAP) family and a substrate for cdk1 [18]. The subsequent decrease in cyclin B1-cdk1 activity results in a decrease in the levels of survivin and increase in sensitivity to induction of apoptosis. Whether this mechanism is generally applicable to paclitaxel-mediated inhibitory effects in different types of prostate cancer cells is not clear.

To better understand how 2-ME and paclitaxel function as anti-prostate cancer agents, we focused on the effect of these drugs on cyclin A and B1 proteins and their associated kinase activities. We report results demonstrating strong similarities of 2-ME with paclitaxel in regard to an increase in cyclin B1 protein and its associated kinase activity followed by a decrease in cyclin A-dependent kinase activity and induction of apoptosis in prostate cancer cells. Inhibition of cyclin B1-dependent kinase activity blocked subsequent induction of apoptosis by both agents. These results indicated that 2-ME inhibition of prostate cancer cells involves several steps in common with paclitaxel, a clinically relevant chemotherapeutic agent for AI-PC.

2. Materials and methods

2.1. Reagents

2-ME, paclitaxel, dimethylsulfoxide (DMSO), lithium chloride, and propidium iodide (PI) were purchased from Sigma (St Louis, MO, USA). Histone H1 protein was purchased from Roche Applied Sciences (Indianapolis, IN, USA). 4′-6-Diamidino-2-phenylindole (DAPI), purvalanol A, alsterpaullone, PD 98059, caspase-3 substrate (Ac-DEVD-*p*NA) and inhibitor (DEVD-CHO) were purchased from Calbiochem (San Diego, CA, USA). Annexin V-FITC was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2. Cell culture and treatment with 2-ME and paclitaxel

Human prostate carcinoma cell lines LNCaP-FGC [19], DU 145 [20], and PC-3 [21] were obtained from the American Type Culture Collection (Rockville, MD, USA). Cultures were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) with 5% fetal bovine serum (Hyclone, Logan, UT, USA),

100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin (Invitrogen). Prostate cancer cells were treated with different doses of 2-ME (0.5–10 μM), paclitaxel (0.5–50 nM), or DMSO (0.1%) control for varying times (4–72 h). In all the experiments, floating and trypsinized attached cells were pooled for further analysis.

2.3. Three-day cell growth assay for 2-ME and paclitaxel

LNCaP (10,000), DU 145 (2500), and PC-3 (3000) cells were seeded in 96-well plates. The next day, fresh media containing different doses of 2-ME (0.1–50 μM) and paclitaxel (0.1–50 nM) or control (0.1% DMSO) were added and cells incubated for 3 days. The CellTiter Aqueous cell proliferation colorimetric method (Promega, Madison, WI) was used to determine cell viability as per manufacturer's instructions. Cell viability was normalized against the vehicle control and the data expressed as a percentage of control from three independent experiments done in triplicate.

2.4. Flow cytometric analysis

Propidium/hypotonic citrate method [22] was used to study cell cycle distribution of 2-ME and paclitaxel treated prostate cancer cells. After harvesting and washing cells with phosphate-buffered saline (PBS), the cell pellets were resuspended in 0.5 ml of PI staining solution (0.1% sodium citrate, 0.03% NP40, and 50 μg/ml PI), vortexed to release nuclei, and DNA distribution histograms generated by analysis of 10,000 nuclei in a Coulter XL flow cytometer. The percentage of cells in the G1, S, and G2/M DNA content were determined by the ModFit program (Verity Software House, Topsham, ME, USA) from 6 to 8 samples analyzed from at least three independent experiments.

2.5. Western blot analysis

Cell pellets were resuspended in NP40 cell lysis buffer (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, protease inhibitor tablet (Roche Applied Sciences), 50 mM NaF, and 0.1 mM $NaVO_4$), lysed by vortex, left on ice for 30 min, centrifuged, and the protein concentrations of the supernatant determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). After separation of 25–50 μg protein by SDS-PAGE, proteins were transferred by electrophoresis to Immobilon-P membrane (Millipore Corp, Bedford, MA, USA) and incubated in 5% non-fat dry milk, PBS, and 0.25% Tween-20 for 1 h. Antibodies specific for cyclin B1 (GNS1), cyclin A (H-432), cdk1 (17), cdk2 (D-12), p53 (DO-1), p21 (C-19), survivin (FL-142), IAP-1 (H-83), IAP-2 (H-85), and actin (C-11) (Santa Cruz Biotechnology) were diluted 1/1000 in 5% non-fat dry milk, PBS, and 0.25% Tween-20 and incubated overnight at 4 °C. Similarly, antibodies specific for poly ADP-ribose polymerase (PARP; C2-10), Bcl-xL (polyclonal) (BD Biosciences Pharmingen, San Diego, CA, USA), and XIAP (Cell Signaling Technology, Beverly, MA) were diluted 1/1500. Membranes were washed in PBS and 0.25% Tween-20 (3×10 min) and incubated with horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG1/2a or anti-rabbit; 1/2000 dilution; Santa Cruz Biotechnology) for 1 h, washed in PBS and 0.25% Tween-20, and analyzed by exposure to X-ray film (X-Omat, Eastman Kodak Co, Rochester, NY, USA) using enhanced chemiluminescence plus (ECL plus, Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Goat polyclonal antibodies specific for actin and horseradish peroxidase-conjugated secondary antibody (anti-goat IgG; 1/2000 dilution; Santa Cruz Biotechnology) were used for protein loading controls. Total proteins were stained with Coomassie blue for an additional protein loading control. X-ray films were scanned using an Epson Perfection 2450 Photo scanner and the pixel intensity measured using UN-SCAN-IT digitizing software, version 5.1 (Silk Scientific Corp., Orem, UT, USA). Changes in protein levels of 2-ME and paclitaxel treated cells was determined by normalizing values to actin and comparing to values of control treated cells ($=1.0$) in at least three different samples analyzed from 2 to 5 independent experiments. To determine the overall levels of Bcl-xL and survivin in LNCaP, DU 145, and PC-3 cells, the scanned bands from the same blot were normalized to scanned total protein ($n=6$, two independent experiments).

2.6. Cyclin B1 and A-dependent kinase assay

Four hundred micrograms of total protein were incubated with 2 μg anti-cyclin A or B1 antibody for 3 h on ice, followed by the addition of 20 μl protein A/G-agarose (Santa Cruz Biotechnology), and incubated overnight at 4 °C. with agitation. Immune-complexes were collected by centrifugation, washed 3× with NP40 cell lysis buffer, 3× with kinase buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM DTT), resuspended in kinase buffer containing 2 μg histone H1 substrate protein, 25 μM ATP, 5 μCi γ^{32}P-ATP, and incubated for 30 min at 30 °C. Reactions were stopped with SDS gel loading buffer, samples electrophoresed on SDS-PAGE, electroblotted to Immobilon P membranes, and analyzed by autoradiography. Coomassie blue staining of membranes revealed similar loading of histone proteins. The histone band was cut out from the paper and ^{32}P measured by scintillation counting. Changes in kinase activity of 2-ME and paclitaxel treated cells was determined by normalizing the ^{32}P-histone values to the scanned H1 protein value and comparing to values of control treated cells (=1.0) in at least three different samples analyzed from 2 to 5 independent experiments.

2.7. p21 immunoprecipitation and cdk2 western blot

Four hundred micrograms of LNCaP total protein were incubated with 2 μg anti-p21 or rabbit IgG antibody for 3 h on ice, followed by the addition of 20 μl protein A/G-agarose, and incubated overnight at 4 °C. with agitation. Immune-complexes were collected by centrifugation, washed 3× with NP40 cell lysis buffer, and analyzed by Western blot using cdk2 antibody.

2.8. Apoptosis assays

For the DAPI staining apoptosis assay, prostate cancer cells were resuspended in 0.6 ml 4% paraformaldehyde/PBS for 15 min, washed with PBS, and resuspended in 0.5 ml of DAPI (1 μg/ml)/PBS for 10 min. Cells were washed with PBS and 10 μl of concentrated cells added on a microscope slide followed by placement of a coverslip. Cells containing densely stained and fragmented chromatin were identified as apoptotic using a Nikon fluorescence microscope with a DAPI filter. The number of apoptotic cells in at least 250 total cells was determined from at least four random microscope fields. Changes in apoptosis from 2-ME and paclitaxel treated prostate cancer cells was determined as percentage of apoptotic cells in at least five different samples from three independent experiments. There was minimal apoptosis detected in control treated cells (<0.5%). For the annexin V apoptosis assay, prostate cancer cells were resuspended in 100 μl annexin binding buffer (10 mM Hepes, pH 7.9; 140 mM NaCl; 2.5 mM $CaCl_2$) followed by the addition of 2.5 μl of annexin V-FITC and 2 μl PI (50 μg/ml) and incubated for 20 min at room temperature. After the addition of 400 μl annexin binding buffer, the cells were read by flow cytometry and the percentage of early apoptotic cells determined by measuring the annexin-FITC positive/PI negative quadrant using WinMDI version 2.8.

2.9. Caspase-3 assay

Prostate cancer cells were resuspended in 50–100 μl ice cold cell lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA), and incubated 5 min on ice. Cells were centrifuged for 10 min at 4 °C and the supernatant stored at −80 °C. Fifty micrograms of cell extract was added to assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol) containing caspase-3 substrate (Ac-DEVD-*p*NA; 200 nM) and incubated at 37 °C. Absorbance at 405 nM was determined using a microtiter plate reader and the changes in caspase-3 activity from 2-ME and paclitaxel treated prostate cancer cells was determined as fold control treated cells (equals 1.0). Addition of caspase-3 inhibitor (DEVD-CHO; 50 nM) was used to confirm specificity.

2.10. Purvalanol A and alsterpaullone cdk inhibitors

The potent cdk inhibitors purvalanol A and alsterpaullone [23,24] were used to investigate the effect on 2-ME and paclitaxel-mediated apoptosis in prostate cancer cells. Dose response experiments determined that 5 μM purvalanol A blocked 2-ME

and paclitaxel-mediated increase in cyclin B1-dependent kinase activity in DU 145 but not LNCaP cells. For alsterpaullone, a dose of 5 μM in LNCaP and 10 μM in PC-3 blocked 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity. The effect of these doses on 2-ME and paclitaxel-mediated apoptosis was determined using the methods described above.

2.11. Statistical analysis

Statistical differences between 2-ME or paclitaxel-treated and control cells were determined by two-tailed Student's *t*-test with $P<0.05$ considered significant.

3. Results

3.1. Differential growth inhibition of prostate cancer cells by 2-ME and paclitaxel

Using a 3-day cell growth assay, we showed that androgen-dependent LNCaP cells were more sensitive to inhibition by 2-ME compared to androgen-independent DU 145 and PC-3 prostate cancer cells (Fig. 1). The half-maximal inhibitory concentrations (IC_{50}) with standard deviations in parenthesis were as follows: LNCaP, 1.35 μM (±0.25); DU 145, 2.0 μM (±0.41); and PC-3, 10.4 μM (±2.64). DU 145 cells were more sensitive to inhibition by paclitaxel compared to LNCaP and PC-3 cells. The IC_{50} for paclitaxel treated cells were as follows: DU 145, 1.53 nM (±0.3); LNCaP, 2.6 nM (±0.5); and PC-3, 7.0 nM (±1.8). Subsequent experiments sought to determine why LNCaP and DU 145 cells were more sensitive to inhibition by 2-ME and paclitaxel compared to PC-3 cells.

3.2. Low doses of 2-ME and paclitaxel increase LNCaP cells in the G1 phase of the cell cycle

To investigate the cell cycle effects of 2-ME compared to paclitaxel, we used flow cytometric analysis after treatment of prostate cancer cells with varying doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h (Fig. 2). Treatment of LNCaP cells with 2 μM 2-ME and 2 nM paclitaxel resulted in a significant (>13%) increase of cells in the G1 and decrease (>30%) in the S phase of the cell cycle. Similar doses of 2-ME and paclitaxel did not cause G1 accumulation in DU 145 (Fig. 2) and PC-3 (result not shown) cells, probably because their G1 cell cycle checkpoints are defective [25,26]. Treatment of all prostate cancer cells with ≥5 μM 2-ME and ≥10 nM paclitaxel resulted in an increase in G2/M with concomitant decrease in G1. These results suggested that lower doses of 2-ME and paclitaxel blocked LNCaP but not DU 145 or PC-3 cells in the G1 phase of the cell cycle.

3.3. Dose-specific changes in cyclins B1 and A in 2-ME and paclitaxel-treated prostate cancer cells

To investigate molecular changes involved in 2-ME and paclitaxel-mediated G1 and G2/M cell

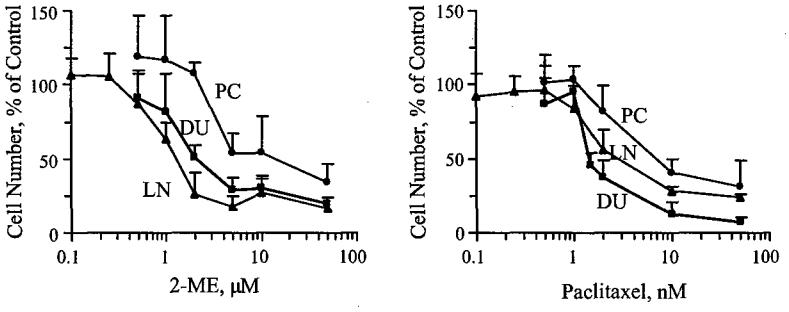

Fig. 1. 2-ME and paclitaxel inhibits growth of human prostate cancer cells in a dose-dependent manner. Androgen-dependent LNCaP (LN, ▲) and androgen-independent DU 145 (DU, ■) and PC-3 (PC, ●) human prostate cancer cell lines were treated with 2-ME (0.1–50 μM), paclitaxel (0.1–50 nM) or vehicle control, as described under Materials and Methods. Cell viability was measured using the CellTiter Aqueous colorimetric method; the data are expressed as percentage of control (mean ± standard deviation) from three independent experiments done in triplicate.

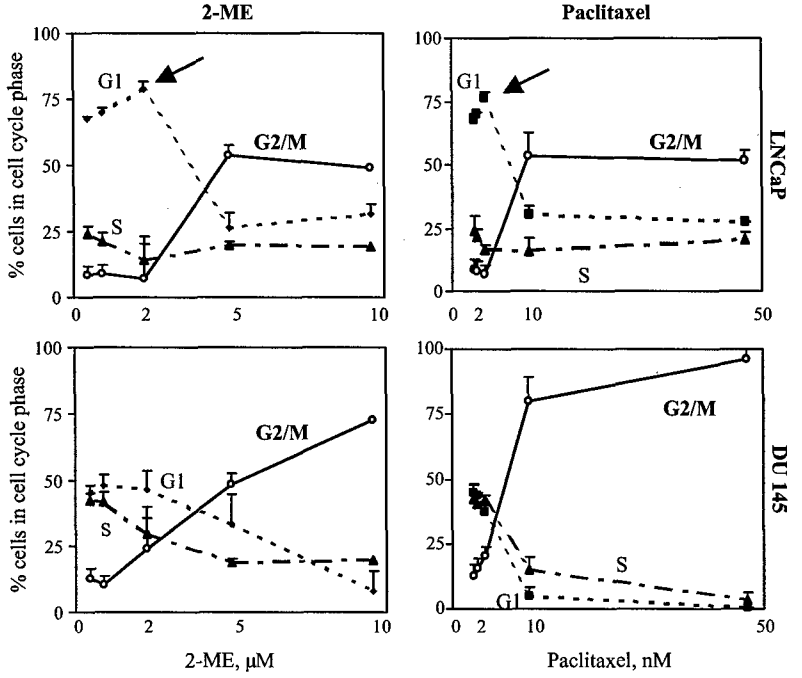

Fig. 2. Flow cytometric analysis of 2-ME and paclitaxel dose response in human prostate cancer cells. LNCaP and DU 145 were treated with varying doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the percentage of cells in the cell cycle phases (G1, S, and G2/M) determined by flow cytometry. In LNCaP, 2 μM 2-ME and 2 nM paclitaxel increased cells in G1 (arrows; $P<10^{-6}$). Higher doses of 2-ME (≥5 μM) and paclitaxel (≥10 nM) blocked cells in G2/M with concomitant reduction in G1. Results are expressed as means ± standard deviation (error bars).

cycle arrest in prostate cancer cells, we analyzed expression of cyclins B1 and A by Western blot and kinase assays (Fig. 3A). Cyclin A protein increases during the S and G2 phase of the cell cycle and is believed to be important for DNA replication [27]. The transition from the G2 to the M phase of the cell cycle requires accumulation of cyclin B1 and activation of its associated kinase, cdk1. The end of the G2/M transition and exit from mitosis requires proteolysis of cyclin B1 and reduction of cdk1 activity [28]. Treatment of LNCaP cells with 5 μM 2-ME and 10 nM paclitaxel for 24 h resulted in a 3–4-fold increase of cyclin B1 protein and its associated kinase activity but no increase in cyclin A protein and its associated kinase activity (Fig. 3A). Similar results were obtained in 2-ME and paclitaxel treated DU 145 and PC-3 cells (Fig. 3B). In LNCaP but not in DU 145 and PC-3 cells, treatment with the G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) resulted in a 4–5-fold decrease in cyclin A-dependent kinase activity without any changes in the levels of cyclin A (Fig. 3A) and cdk2 protein (not shown). These results indicated that (1) the G2/M-promoting doses of 2-ME (≥5 μM) and paclitaxel (≥10 nM) increased cyclin B1 protein and kinase activity to similar levels in LNCaP, DU 145, and PC-3 cells and (2) the G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) inhibited cyclin A-dependent kinase only in LNCaP and may explain why LNCaP cells increased in G1 and decreased in S.

3.4. Time-dependent effects of the G2/M-promoting doses of 2-ME and paclitaxel on cell cycle distribution

To analyze the time-dependent effects on the cell cycle after treatment with 2-ME and paclitaxel over 4, 24, 48, and 72 h, we chose the G2/M-promoting dose of 2-ME (5 μM) and paclitaxel (10 nM). After 4 h of 2-ME treatment, there was a significant increase in cells with G2/M DNA content in DU 145 but not in

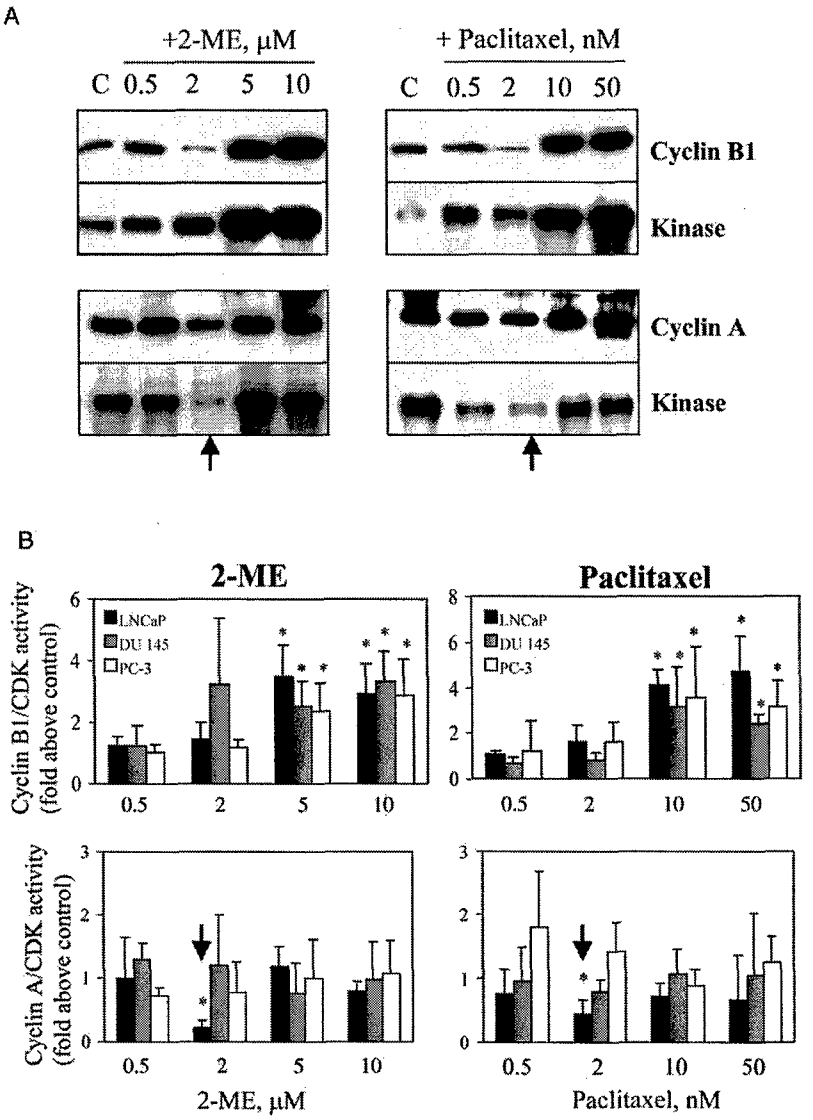

Fig. 3. Effect of G1- and G2/M-promoting doses of 2-ME and paclitaxel on cyclin B1 and A proteins and their associated kinase activities. (A) LNCaP cells were treated with increasing doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the levels of cyclin B1 and A proteins determined by Western blot analysis, normalized to actin protein (not shown), and compared to control (C) treated cells. Cyclin B1 and A proteins were immunoprecipitated and their associated kinase activities (^{32}P-histone) determined from the same lysates. G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) decreased cyclin A-dependent kinase activity (arrow) and the G2/M-promoting doses (5 μM 2-ME and 10 nM paclitaxel) increased cyclin B1-dependent kinase activity. (B) G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) resulted in similar increases in cyclin B1-dependent kinase activity in LNCaP, DU 145, and PC-3 cells compared to control treated cells (*, $P<0.03$). In LNCaP cells, G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) inhibited cyclin A-dependent kinase (arrows). Results are expressed as means (fold control = 1) ± standard deviation (error bars).

LNCaP (Fig. 4). All cells treated with 2-ME and paclitaxel accumulated in G2/M after 24 h with a concomitant decrease in G1. In LNCaP, there was a significant decrease in G2/M after 48 and 72 h treatment with 2-ME and paclitaxel. In DU 145 (Fig. 4) and PC-3 (result not shown), however, cells remained blocked in G2/M after treatment for 48 and 72 h with 2-ME but not paclitaxel. After the initial

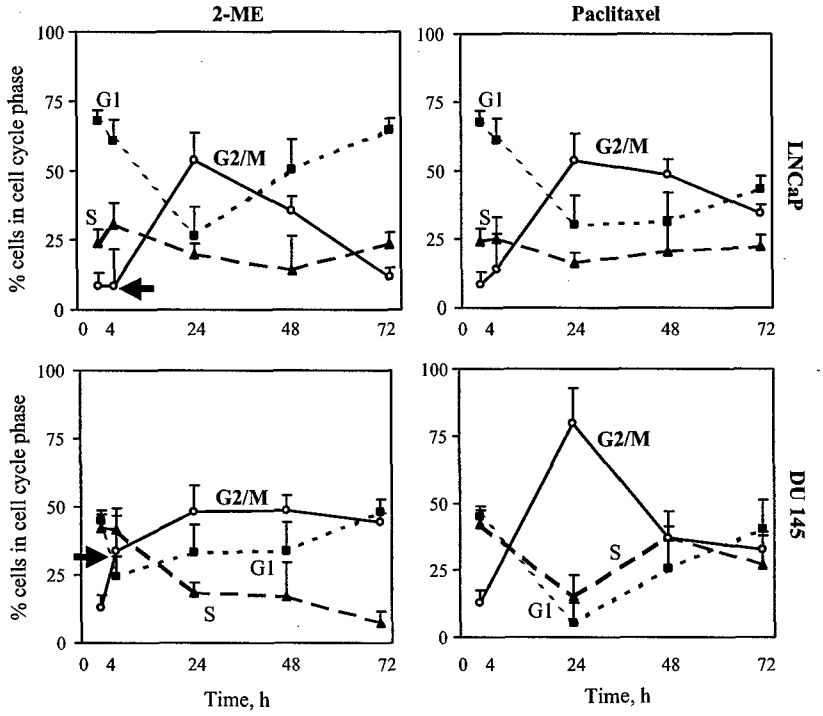

Fig. 4. Changes in cell cycle distribution with time after treatment of prostate cancer cells with 2-ME and paclitaxel. LNCaP and DU 145 cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the percentage of cells in the cell cycle phases (G1, S, and G2/M) analyzed by flow cytometry. In 2-ME treated DU 145 but not LNCaP cells, there was a significant increase in G2/M after 4 h (arrow; $P < 0.03$). At 24 h, all cells showed increase in G2/M with a concomitant reduction in G1. At 48 and 72 h, there was a decrease in G2/M, except with 2-ME treated DU 145 cells. Results are expressed as means ± standard deviation (error bars).

decrease of LNCaP cells in G1 at 24 h, there was a significant increase after 48 and 72 h in 2-ME treated cells. Although these results revealed a common G2/M block at 24 h, there were differences in the cell cycle distribution at 48 and 72 h between 2-ME and paclitaxel-treated prostate cancer cells.

3.5. Changes in cyclins B1 and A before and after 2-ME and paclitaxel-mediated block in G2/M

Since all prostate cancer cells were blocked at G2/M after 24 h treatment with ≥5 μM 2-ME and ≥10 nM paclitaxel, it was not surprising that there was a marked accumulation of cyclin B1 protein (Figs. 2 and 3). However, there was a significant increase in cyclin B1 protein and its associated kinase in LNCaP cells treated with 2-ME after only 4 h, a time when there was no increase in cells with G2/M DNA content (Figs. 4 and 5). This suggested that the increase of cyclin B1 protein and kinase activity was not simply due to increase in the G2/M fraction. In general, the levels of cyclin B1 protein peaked at 24 h and decreased at 48 and 72 h after treatment with 2-ME and paclitaxel (Fig. 5A). Cyclin B1-dependent kinase activity also peaked at 24 h and decreased to control levels by 72 h with the exception of LNCaP cells treated with paclitaxel, in which activity remained significantly elevated. These results indicated that there were differences in the later (>24 h) effects of 2-ME and paclitaxel on cyclin B1-dependent kinase activity after the initial prolonged activation at 24 h. There was a 2–5-fold decrease in cyclin A protein and its associated kinase activity in all prostate cancer cell lines at 48 and 72 h, a time when apoptotic cells were increased (see Fig. 7A).

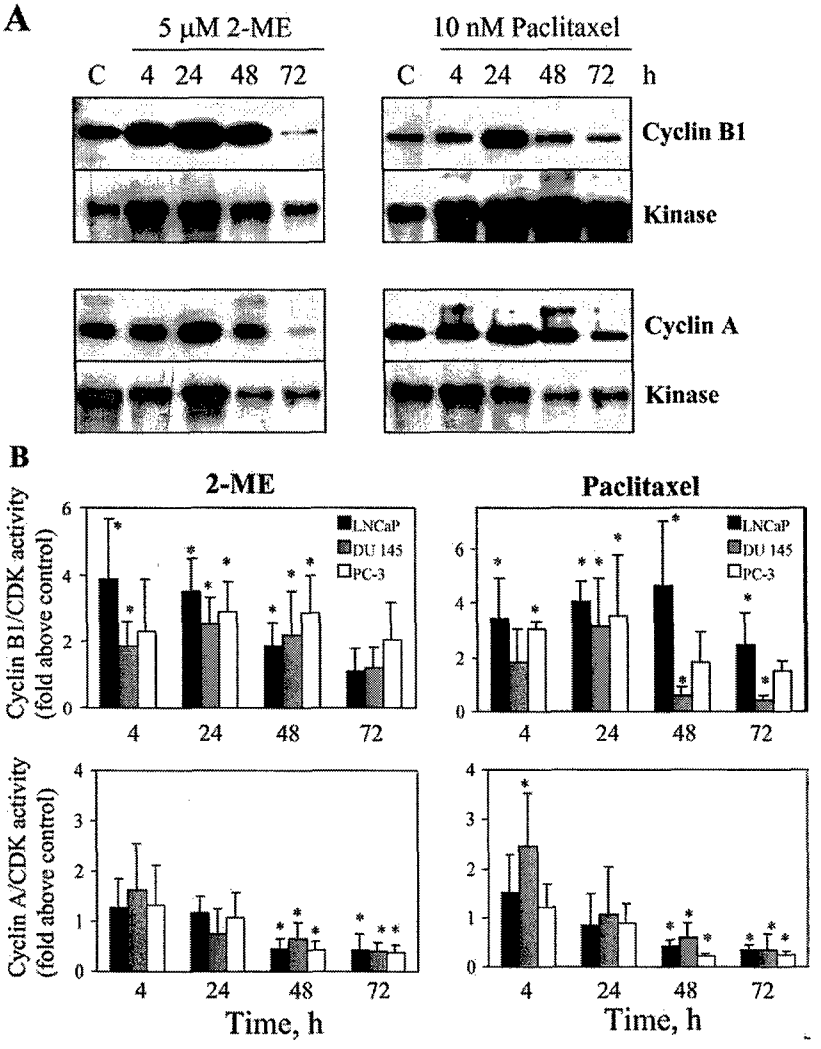

Fig. 5. Changes in cyclin B1 and A proteins and their associated kinase activities with time after treatment of prostate cancer cells with 2-ME and paclitaxel. (A) LNCaP cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the levels of cyclin B1 and A proteins analyzed by Western blot and compared to control (C) treated cells. Cyclin B1 and A proteins were immunoprecipitated and their associated kinase activities (^{32}P-histone) determined from the same lysates. Cyclin B1-dependent kinase activity decreased in 2-ME but not paclitaxel treated LNCaP cells at 48 and 72 h. Cyclin A-dependent kinase activity decreased in both 2-ME and paclitaxel treated LNCaP cells at 48 and 72 h. (B) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in a peak of cyclin B1-dependent kinase activity at 24 h with subsequent decline at 48 and 72 h. The exception is paclitaxel treated LNCaP cells, which maintained high cyclin B1-dependent kinase activity at 48 and 72 h despite low cyclin B1 protein. There was a greater decrease in cyclin B1-dependent kinase activity in paclitaxel compared to 2-ME treated DU 145 cells (*, $P<0.05$). Cyclin A-dependent kinase activity decreased in all 2-ME and paclitaxel treated prostate cancer cells (*, $P<0.04$) at 48 and 72 h. Results are expressed as means (fold control = 1) ± standard deviation (error bars).

3.6. p53 and p21 proteins are increased in LNCaP cells treated with 2-ME and paclitaxel

To further investigate molecular changes involved in 2-ME and paclitaxel-mediated G1 and G2/M cell cycle arrest in LNCaP cells, we analyzed expression of p53 and p21 by Western blot (Fig. 6). The G1-promoting doses of 2-ME (2 μM) and paclitaxel (2 nM) that decreased cyclin A-dependent kinase activity (see Fig. 3) resulted in a 3–4-fold increase in

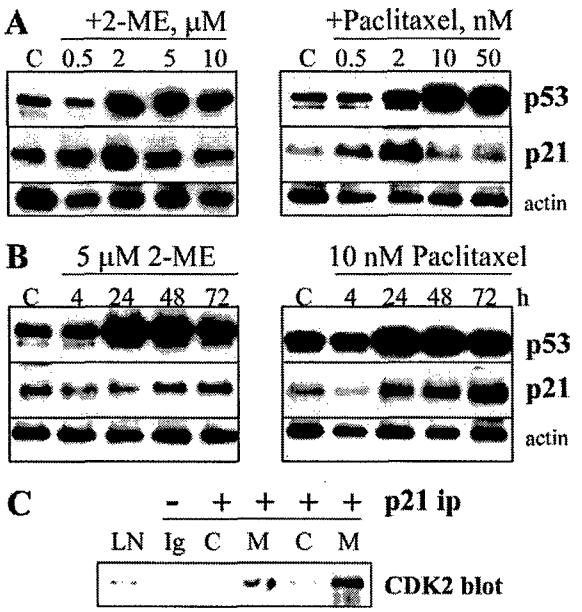

Fig. 6. 2-ME and paclitaxel increased p53 and p21 proteins in LNCaP cells. (A) LNCaP cells were treated with increasing doses of 2-ME (0.5–10 μM) and paclitaxel (0.5–50 nM) for 24 h and the levels of p53 and p21 proteins determined by Western blot, normalized to actin, and compared to control (C) treated cells. p53 increased with 2-ME (≥2 μM) and paclitaxel (≥2 nM), whereas p21 significantly increased with only 2 μM 2-ME and 2 nM paclitaxel ($P<0.05$). (B) LNCaP cells were treated with 5 μM 2-ME and 10 nM paclitaxel for 4, 24, 48, and 72 h and the levels of p53 and p21 proteins analyzed by Western blot. p53 increased at 24 h and remained elevated at 48 and 72 h, whereas p21 increased at 48 and 72 h. (C) Increased association of p21 with cdk2 protein in 2-ME treated LNCaP cells. p21 protein was immunoprecipitated (+ ip) from LNCaP cells treated with 5 μM 2-ME (M) or control (C) for 24 h and samples analyzed by Western blot for cdk2 protein. Duplicate samples from C and M are shown. Positive control was LNCaP (LN) cell total lysate and negative control was immunoprecipitation with non-specific rabbit Ig (-ip).

p53 protein (Fig. 6A); p53 is mutated in DU 145 cells and PC-3 cells and is non-functional [26]. Similarly, p53 protein levels were increased at 24 h (but not at 4 h) using the G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) and remained elevated at 48 and 72 h (Fig. 6B). Because p53 is known to increase transcription of the cdk inhibitor p21 gene [29], we also analyzed expression of p21 protein by Western blot. The G1-promoting dose of 2-ME (2 μM) and paclitaxel (2 nM) resulted in a small but significant two-fold increase in p21 protein, whereas the G2/M-promoting doses did not increase p21 (Fig. 6A). There was an increased association of p21 with cdk2 when the G1-promoting dose of 2-ME (2 μM) was utilized (Fig. 6C), suggesting a mechanism for inhibition of cyclin A-dependent kinase and blocking of LNCaP cells in G1. At 48 and 72 h, there was a 2–6-fold increase in p21 protein and this correlated with decreased cyclin A-dependent kinase activity (Figs. 5 and 6). The levels of p21 were very low in DU 145 and PC-3 cells and did not change with 2-ME and paclitaxel treatment (result not shown).

3.7. Increased apoptosis in prostate cancer cells after 2-ME and paclitaxel-mediated mitotic block

To analyze the time of the appearance of apoptotic cells relative to G2/M block, we performed DAPI staining and caspase-3 assays on prostate cancer cells treated with 2-ME (5 μM for LNCaP and DU 145; 10 μM for PC-3) and paclitaxel (10 nM) for 24, 48, and 72 h (Fig. 7A). In LNCaP, there was a significant increase of apoptotic cells from 6, 16, and >25% after 24, 48, and 72 h treatment with 2-ME and paclitaxel, which also corresponded with a significant increase in caspase-3 activity (Fig. 7A). Less apoptotic cells and caspase-3 activity were identified in 2-ME treated DU 145 and PC-3 cells compared to LNCaP cells, probably explaining the differential growth inhibition (LNCaP > DU 145 > PC-3). A difference was observed in paclitaxel treated DU 145 cells, where there were greater number of apoptotic cells (6.5, 25, 27%) compared to 2-ME treated cells (2, 9, 13%). However, there was not a corresponding increase in caspase-3 activity in paclitaxel treated DU 145 cells, possibly due to caspase-independent events or other caspases were more active.

Similar results were obtained in DU145 and PC-3 using annexin V-FITC/PI flow cytometric detection of early apoptotic cells (Fig. 7B). The greater amount of apoptosis measured with annexin V compared to DAPI probably reflected later events in the apoptotic pathway (chromatin fragmentation/condensation) that were inhibited compared to earlier events (annexin V binding). In PC-3 cells (least sensitive to 2-ME and paclitaxel), longer treatment (6 days) was required to obtain increased number of apoptotic cells (8%) (see Fig. 9B). These results indicated that the 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells predominantly occurred after

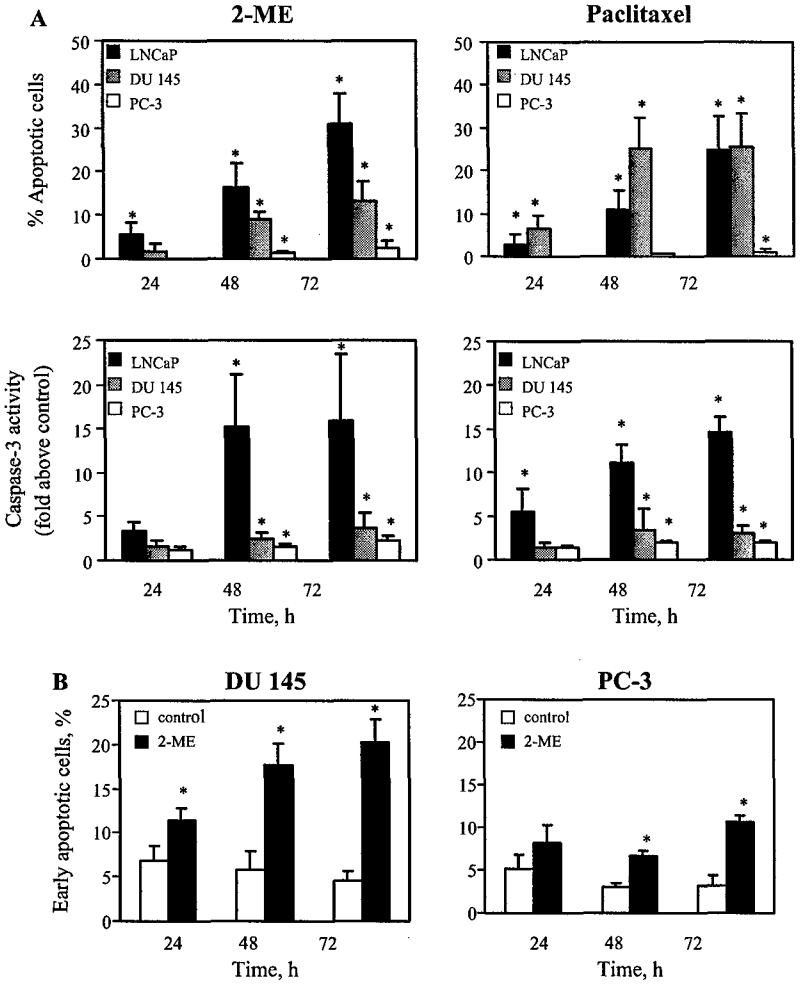

Fig. 7. 2-ME and paclitaxel increased apoptosis and caspase-3 activity in prostate cancer cells at 48 and 72 h. (A) 2-ME and paclitaxel treatment of LNCaP, DU 145, and PC-3 cells resulted in significantly increased apoptotic cells (DAPI) and caspase-3 activity at 48 and 72 h (*, $P<0.04$). Increased apoptotic cells and caspase-3 activity was greatest in LNCaP cells. In DU 145 cells, paclitaxel increased apoptotic cells greater than 2-ME at 48 and 72 h. There was minimal apoptosis (<0.5%) detected by DAPI in the control treated cells (not shown). Caspase-3 results are expressed as means (fold above control = 1) ± standard deviation (error bars). (B) Flow cytometric analysis of annexin V-FITC and PI stained DU 145 and PC-3 cells after treatment with 2-ME (5 μM for DU 145 and 10 μM for PC-3) for 24, 48, and 72 h compared to control cells ($n=6$; *, $P<0.005$). Early apoptotic cells represent the annexinV-FITC positive and PI negative population of cells.

the G2/M cell cycle block at 24 h. In contrast, treatment of LNCaP cells with G1-promoting doses (2 μM 2-ME and 2 nM paclitaxel) resulted in minimal (<1.5%) induction of apoptosis at all time points (not shown). In addition, blocking LNCaP and DU 145 cells in the S phase with 1 μM aphidicolin reduced 2-ME-mediated induction of apoptosis (result not shown), suggesting that G2/M block by 2-ME is important for induction of apoptosis.

3.8. 2-ME and paclitaxel decrease in XIAP correlates with increased apoptosis in prostate cancer cells

To investigate why 2-ME and paclitaxel-treated LNCaP cells undergo apoptosis greater than DU 145 and PC-3 cells, we sought to identify differences in the levels of proteins important in apoptosis by Western blot analysis. Cleavage of the PARP protein, indicative of apoptosis, occurred to a greater extent in 2-ME and

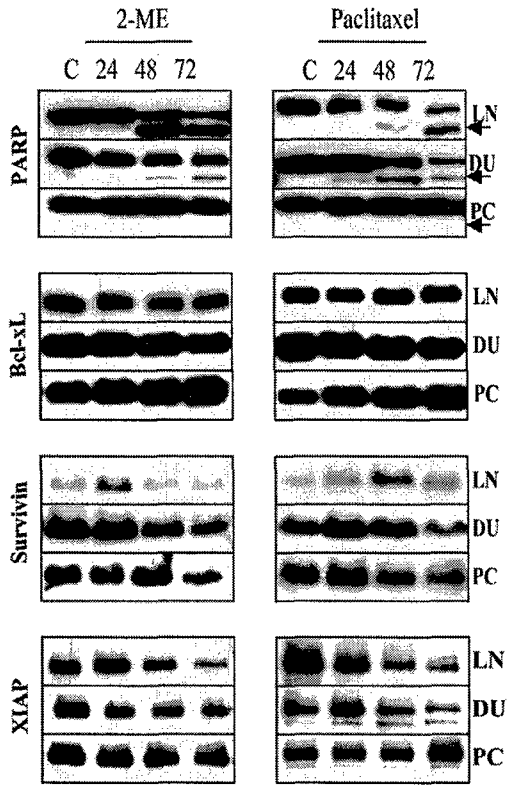

Fig. 8. 2-ME and paclitaxel decrease the levels of IAP protein XIAP at 48 and 72 h. LNCaP (LN), DU 145 (DU), and PC-3 (PC) cells were treated with 5–10 μM 2-ME and 10 nM paclitaxel for 24, 48, and 72 h and the levels of PARP, Bcl-xL, survivin, and XIAP proteins determined by Western blot, normalized to actin (not shown), and compared to control (C) treated cells. There was increased PARP cleavage (arrow) in LNCaP and DU 145 compared to PC-3 cells. There was greater cleavage of PARP in paclitaxel compared to 2-ME treated DU 145 cells. No significant differences were notable in Bcl-xL. Mitotic block and increased cyclin B1-dependent kinase activity resulted in increased survivin, which decreased at 72 h. 2-ME and paclitaxel decreased XIAP in LNCaP cells at 48 and 72 h. Paclitaxel but not 2-ME decreased XIAP in DU 145 cells at 48 and 72 h. There were no differences in XIAP levels in 2-ME treated DU 145 cells and 2-ME and paclitaxel treated PC-3 cells. The overall levels of Bcl-xL and survivin were greater in DU 145 and PC-3 compared to LNCaP cells.

paclitaxel treated LNCaP and DU 145 compared to PC-3 cells (Fig. 8). There were no differences in the levels of the anti-apoptotic protein Bcl-xL. The levels of the anti-apoptotic protein survivin increased when the cyclin B1-cdk1 activity was elevated and subsequently decreased to a level similar to or below that of control cells (Fig. 8). 2-ME and paclitaxel increase in apoptosis in LNCaP (and in paclitaxel treated DU 145) correlated with a 2–3-fold decrease in the levels of XIAP, a member of the IAP family, at 48 and 72 h [30] (Fig. 8). There were no changes in the levels of other IAP family members IAP-1 and IAP-2 (result not shown). Another potential reason for the differential sensitivity to apoptosis may be the 1.5–2-fold higher levels of the Bcl-xL and survivin proteins in DU 145 and PC-3 compared to LNCaP cells, which may have protected these cells from 2-ME and paclitaxel-mediated induction of apoptosis.

3.9. Cyclin-dependent kinase inhibitors block 2-ME and paclitaxel-mediated induction of apoptosis

To investigate whether the increase in cyclin B1-dependent kinase activity was required for 2-ME and paclitaxel-mediated induction of apoptosis, we utilized the potent cdk inhibitors purvalanol A and alsterpaullone [23,24]. Treatment of LNCaP cells with 5 μM alsterpaullone and DU 145 cells with 5 μM purvalanol A for 24 h resulted in an increase in G2/M (result not shown) and blocked the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity (Fig. 9A). In addition to inhibiting cyclin B1-dependent kinase activity, alsterpaullone also decreased cyclin A-dependent kinase in control and 2-ME and paclitaxel-treated LNCaP cells. In contrast, purvalanol A, which has a higher specificity for the inhibition of cyclin B1-dependent kinase, increased cyclin A-dependent kinase in control and 2-ME and paclitaxel-treated DU 145 cells (Fig. 9A). At 72 h, alsterpaullone and purvalanol A blocked 2-ME and paclitaxel-mediated induction of apoptosis in LNCaP and DU 145 cells, as determined by DAPI assay, caspase-3 activity, and PARP cleavage (Fig. 9B and C). Because treatment of PC-3 cells with 2-ME and paclitaxel induced minimal apoptosis at 72 h (1–2%; Fig. 7A), we chose treatment for 6 days when apoptosis increased to $>8\%$. The results showed that 10 μM alsterpaulone similarly blocked the 2-ME and paclitaxel increase of apoptosis in PC-3 cells. Kinase inhibitors PD 98059 (20 μM; MAP kinase inhibitor) and lithium chloride (30 mM; glycogen synthase kinase 3β inhibitor) did not significantly block 2-ME-mediated induction of apoptosis in LNCaP and DU 145 cells (result not shown). These results suggested that 2-ME and paclitaxel-mediated increase

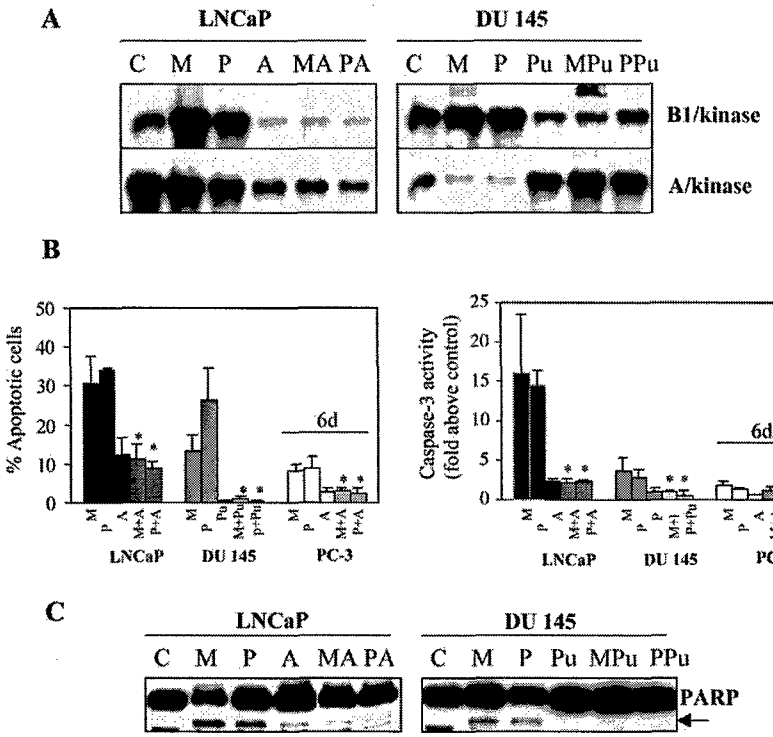

Fig. 9. Inhibition of the 2-ME and paclitaxel-mediated increase of cyclin B1-dependent kinase activity blocked induction of apoptosis. (A) LNCaP and DU 145 cells were treated for 24 h with 5–10 μM 2-ME (M), 10 nM paclitaxel (P), 5 μM alsterpaullone (A, LNCaP), 5 μM purvalanol A (Pu, DU 145), and 2-ME or paclitaxel combined with alsterpaullone (MA, PA) and purvalanol A (MPu, PPu). Cyclin B1 and A proteins were immunoprecipitated and the associated kinase activity (B1/kinase and A/kinase) determined and compared to control (C) treated cells. In LNCaP, alsterpaullone decreased both cyclin B1- and A-dependent kinase activity, whereas in DU 145 purvalanol A decreased cyclin B1-dependent kinase activity with a concomitant increase in cyclin A-dependent kinase activity. (B) Cdk inhibitors alsterpaullone and purvalanol A significantly inhibited 2-ME and paclitaxel-mediated apoptosis in LNCaP, DU 145 (72 h), and PC-3 (6 days) as determined by DAPI staining (left panel) and caspase-3 activity (right panel) (*, $P<0.01$). (C) Western blot showing that alsterpaullone and purvalanol A blocked 2-ME and paclitaxel-mediated cleavage of PARP in LNCaP and DU 145 cells at 72 h. A non-specific band was noted below the cleaved PARP fragment.

in cyclin B1-dependent kinase activity was required for induction of apoptosis in prostate cancer cells.

4. Discussion

We analyzed in human prostate cancer cells the effects of the promising chemotherapeutic drugs 2-ME and paclitaxel on the cyclin proteins important in the G2/M phase of the cell cycle. Our results suggested a requirement for G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) to increase cyclin B1-dependent kinase activity in order to induce apoptosis. Furthermore, our results suggested that androgen-dependent LNCaP cells were sensitive to inhibition by 2-ME and paclitaxel because lower drug doses increased p53 and p21 proteins, inhibited cyclin A-dependent kinase activity, and resulted in a G1 block. In addition, G2/M-promoting doses of 2-ME and paclitaxel inhibited cyclin A-dependent kinase activity in all prostate cancer cells at the time apoptosis was increased. However, the differential induction of apoptosis by 2-ME and paclitaxel in prostate cancer cells is correlated with the ability to reduce the levels of XIAP, a member of the IAP family that inhibits caspase activity [30]. Overall, our results indicated that 2-ME has a similar mechanism as paclitaxel in the effects on the cell cycle and induction of apoptosis of human prostate cancer cells.

Similar to paclitaxel treatment of prostate cancer cells, 2-ME increased cyclin B1 protein and blocked cells in mitosis [14–16]. Our results showed that the increase in cyclin B1 protein and kinase activity in LNCaP cells occurred before significant changes in the cell cycle distribution (Figs. 4 and 5). This suggests that it is not just the mitotic spindle checkpoint that increases cyclin B1 protein and kinase activity. Our data agrees with data obtained from paclitaxel treatment of breast and epidermal cancer cells showing that increase of cyclin B1 protein and its associated cdk is required for induction of apoptosis [14,15]. The mechanism proposed for the paclitaxel-mediated increase in cyclin B1 protein is by inhibition of the proteosomal degradation system, which is a key component in the reduction of cyclin B1 protein levels required for metaphase to anaphase transition during mitosis [31]. The end result of increasing cyclin B1 protein is the increase of its associated cdk1 activity, which has been shown to be important in the induction of mitotic catastrophe and many forms of apoptosis [32].

An issue is whether 2-ME and paclitaxel-mediated induction of apoptosis in prostate cancer cells requires the initial increase of cyclin B1-dependent kinase or the subsequent reduction of cyclin B1-dependent kinase activity. The decrease of cyclin B1-dependent kinase activity is proposed to cause apoptosis in sensitive cells by reducing the levels of survivin, a member of the IAP family of proteins and a substrate for cdk1 [17]. Despite the similar decreased levels of cyclin B1-dependent kinase activity in 2-ME treated prostate cancer cells, however, there was a greater induction of apoptosis in LNCaP compared to DU 145 and PC-3 cells (Figs. 5 and 7). In addition, LNCaP cells treated with paclitaxel had elevated cyclin B1-dependent kinase activity at a time when apoptosis was increased. In contrast, DU 145 cells treated with paclitaxel resulted in a faster exit from mitotic block and lower levels of survivin at 72 h, possibly contributing to the greater induction of apoptosis compared to 2-ME treated DU 145 cells (Figs. 4, 7 and 8). These results indicated that decreased cyclin B1-dependent kinase activity and exit from mitotic block varied between 2-ME and paclitaxel treatment of prostate cancer cells. Therefore, we conclude that the initial 2-ME and paclitaxel-mediated increase in cyclin B1-dependent kinase activity is more important than the subsequent decrease in activity for the induction of apoptosis. The substrates for cyclin B1-dependent kinase in addition to survivin that may mediate this effect are yet to be identified.

A common mechanism for chemotherapeutic drug inhibition of cancer cells is the increase in p53 and p21 proteins and block in the G1 phase of the cell cycle [33]. Our studies showed that a similar mechanism was also evident using lower doses of 2-ME and paclitaxel in LNCaP cells, which inhibited cyclin A-dependent kinase activity. Similar results were obtained in p53 wild type lung and breast cancer cell lines treated with 3–6 nM paclitaxel [34]. In order to maximize induction of apoptosis, however, G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) that increased cyclin B1-dependent kinase activity were required. In addition, the G2/M-promoting doses of 2-ME (5 μM) and paclitaxel (10 nM) inhibited cyclin A-dependent kinase activity at a time when apoptosis was maximized at 48 and 72 h. An important role for inhibition of cdk2 and induction of apoptosis in cancer but not normal cells was reported [35]. However, a recent report casts doubt on the importance of cdk2 inhibition in cancer therapy [36]. The cdk inhibitor purvalanol A as a single drug inhibited cyclin B1—but not cyclin A-dependent kinase activity in DU 145 cells, resulting in minimal apoptosis. Purvalanol A combined with 2-ME or paclitaxel blocked induction of apoptosis, indicating the importance of increased cyclin B1-dependent kinase activity (Fig. 9). In contrast, alsterpaullone as a single drug inhibited both cyclin B1- and cyclin A-dependent kinase activity and induced apoptosis in LNCaP cells. However, alsterpaullone blocked the 2-ME or paclitaxel-mediated induction of apoptosis (Fig. 9). We suggest that in addition to the early activation of cyclin B1-dependent kinase activity, later inhibition of cyclin A-dependent kinase activity plays an important role in 2-ME and paclitaxel inhibition of prostate cancer cells.

The effects of 2-ME and paclitaxel on cyclin B1- and cyclin A-dependent kinase activity cannot explain the differential induction of apoptosis in prostate cancer cells (LNCaP ≥ DU 145 > PC-3). It is likely that the expression of wild type p53 in LNCaP but not in DU 145 and PC-3 cells plays an important role in the greater induction of apoptosis. An important role for p53 in 2-ME-mediated apoptosis has been demonstrated for LNCaP and lung cancer cells [10,37]. The ability of 2-ME

and paclitaxel to decrease the levels of the IAP family member XIAP at 48 and 72 h correlated with increased apoptosis (Figs. 7 and 8). Inhibition of XIAP has been shown to increase apoptosis in cancer cells either directly or indirectly by sensitizing to other chemotherapeutic drugs [38,39]. A predominant role for members of the IAP family in the regulation of the induction of apoptosis in prostate cancer cells has been proposed [40]. Therefore, drugs that decrease the levels of anti-apoptotic proteins like XIAP should shift the overall balance towards apoptosis even in the most resistant AI-PC cells. In addition, higher levels of the anti-apoptotic proteins Bcl-xL and survivin in DU 145 and PC-3 compared to LNCaP may contribute to the differential induction of apoptosis by 2-ME and paclitaxel. Overexpression of Bcl-xL has a well established role as a powerful anti-apoptosis factor in prostate cancer and inhibition of Bcl-xL by anti-sense oligonucleotides can sensitize PC-3 cells to drug-mediated apoptosis [41,42].

In summary, our studies indicate that 2-ME is similar to paclitaxel in the early activation of cyclin B1- and later inhibition of cyclin A-dependent kinase activity and this may be an important mechanism for induction of apoptosis in prostate cancer cells. We are currently investigating in a transgenic mouse model of prostate cancer [43] whether these molecular changes are also occurring in vivo. Anti-cancer chemotherapeutic agents ideally should take advantage of the molecular differences between transformed and normal cells and induce apoptosis only in cancer cells. Two such differences may be the overexpression of cyclin B1 protein in cancer cells [44] and the differential sensitivity of cancer cells to the inhibition of cyclin A-dependent kinase [35]. We suggest that 2-ME and paclitaxel take advantage of these differences to inhibit the growth of prostate cancer cells and induce apoptosis. Given that paclitaxel has an effect on patients with AI-PC as a single drug and in combination with other drugs [45], our results hold promise that 2-ME will have a similar efficacy in AI-PC.

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